Hepatitis C virus (HCV) is a human hepatotropic virus, but the relevant host factors restricting HCV infection to hepatocytes are only partially understood. We demonstrate that exogenous expression of defined host factors reconstituted the entire HCV life cycle in human nonhepatic 293T cells. This study shows robust HCV entry, RNA replication, and production of infectious virus in human nonhepatic cells and highlights key host factors required for liver tropism of HCV.

Virus-host interactions that determine and restrict specific tissue and host tropisms have a complex evolutionary history and also have significant consequences for the pathogenesis of viral infection and human disease. Viral hepatitis is a major disease burden. Indeed, infection of hepatocytes by a variety of hepatotropic viruses from different orders and families can lead to tissue inflammation, fibrosis, and hepatocellular carcinoma. Hepatitis C virus (HCV), a member of the family Flaviviridae, is a prime example of a virus that causes chronic hepatitis worldwide. While HCV primarily infects hepatocytes of humans and chimpanzees, the virus has been shown to enter neuronal and endothelial cells of the blood-brain barrier. However, infection of these cells occurs at a low level, and production of infectious viruses is greatly diminished relative to that in liver-derived cells (9, 10). Unlike HCV, other members of the family Flaviviridae have a much broader tissue and species tropism. For example, dengue virus infects and replicates both in the midgut epithelia of Aedes aegypti mosquitoes and in human monocytes and hepatocytes (20, 25, 39). Moreover, a virus closely related to HCV was recently identified from respiratory samples from dogs (18). A large panel of host factors required for HCV has been identified so far (36). However, the key host factors mediating liver tropism of the virus and allowing reconstitution of the viral life cycle in human cells are still only partially understood.

Taking advantage of our current knowledge of host factors involved in HCV infection, we sought to engineer a human kidney cell line (293T) that would be capable of sustaining the entire HCV life cycle. The aim was to define host factors that are necessary and sufficient for the HCV life cycle, in order to understand the liver tissue specificity of HCV.

293T cells were obtained from ATCC and their identity was verified by genomic profile comparison to the LGC Standards database by short tandem repeat profiling as described previously (1) (Fig. 1A). In order to render them infectible by HCV, we used lentiviral vectors to express the four principal HCV host entry factors—claudin-1 (CLDN1), CD81, occludin (OCLN), and scavenger receptor class B type I (SR-BI) (2, 7, 34, 35)—by using previously described expression constructs and methods (3, 24). Four stable 293T cell lines were selected to express either CLDN1 alone, CD81/OCLN with or without CLDN1, or CLDN1/CD81/OCLN together with SR-BI (293T-4R). After verifying stable expression of these proteins using receptor-specific antibodies (Fig. 1B), we infected these cells with HCV pseudoparticles expressing the envelope glycoproteins of HCV genotype 1b (HCVpp; HCV-J strain described in reference 31). While CLDN1 expression alone conferred limited permissiveness for HCV infection, as previously described (7), expression of all four factors enhanced HCV entry to a level that was around 4-fold higher than that in Huh7.5.1 cells, which is the liver-derived model hepatoma cell line for studying HCV infection (Fig. 1C).

Genuine cell culture infection of HCV (HCVcc) was then investigated in 293T-4R cells using a chimeric virus composed of two genotype 2a isolates (designated Jc1 [19, 32]) and engineered for Renilla luciferase expression (JcR2a [38]). However, as shown in Fig. 2A, overcoming the HCV entry block was not sufficient for robust viral RNA replication in 293T cells.

Several studies have shown that micro-RNA 122 (miR122) is a liver-specific host factor critical for HCV replication (5, 16, 17, 28). Since Northern blot analyses demonstrated undetectable miR122 expression in 293T-4R cells (Fig. 2C), we investigated whether exogenous miR122 expression reconstituted viral RNA replication. Indeed, stable expression of this factor, by using miR122-encoding lentiviruses in the 293T-4R line, rendered the cells permissive for bona fide HCVcc infection, with replication to levels comparable to those seen with Huh7.5.1 cells, as assessed by luciferase reporter activity (Fig. 2B). Further confirmation of genuine infection was obtained by observing similar infectivity (determined as 50% tissue culture infective doses [TCID50]) with HCVcc (Jc1) without a reporter gene, by detecting expression of viral protein NS5A (Fig. 2B). We verified expression of miR122 in transduced 293T-4R/miR122 cells, and the level was comparable...
to that in Huh7.5.1 cells, as assessed by Northern blotting (Fig. 2C), and the cell proliferation rates of the different cell lines were similar (data not shown). Kinetics of HCV replication in 293T-4R/miR122 cells matched those of Huh7.5.1 cells, suggesting that aside from miR122, cell factors present in human liver- and kidney-derived cells are equally efficient for replication, as assayed by luciferase reporter gene expression (Fig. 2D). Expression of viral proteins in infected cells was further confirmed using HCV core-specific immunofluorescence (Fig. 2E) and flow cytometry (data not shown).

To further confirm whether viral entry and replication in stably transduced 293T cells are mediated by the same host and virus factors as in human Huh7.5.1 hepatoma cells, we used well-characterized entry and replication inhibitors. Antibodies directed against the HCV entry factors CD81 (JS-81; BD Biosciences), CLDN1 (11), and SR-BI (M. N. Zahid, M. Turek, F. Xiao, V. L. D. Thi, M. Guérin, I. Fofana, P. Bacheiller, J. Thompson, L. Delang, J. Neyts, D. Bankwitz, T. Pietschmann, M. Dreux, F.-L. Cosset, F. Grunert, T. F. Baumert, and M. B. Zeisel, submitted for publication) recognizing indicated entry factors. Entry factor-transduced cells (dark gray histograms) were compared to naive 293T cells (light gray histograms) and isotype control antibody (catalog no. 10400C; Life Technologies) (white histograms with dashed lines). The x axis shows fluorescence intensity; the y axis shows the number of events. (C) Transduced 293T cells were assessed for HCVpp (genotype 1b; HCV-J strain; produced as described in reference 31) entry by determining luciferase activity 72 h postinfection as previously described (35). Results were first normalized to vesicular stomatitis virus pseudoparticle entry (VSV-Gpp; produced as described in reference 29) and a monoclonal antibody recognizing the low-density-lipoprotein (LDL) receptor binding domain of ApoE (37) effectively neutralized HCV infection of 293T-4R/miR122 cells (Fig. 2F). The same was true for the recently identified HCV entry inhibitor erlotinib, which targets the kinase activity of the host entry regulatory protein, epidermal growth factor receptor (EGFR) (Fig. 2F) (24). Likewise, the well-characterized inhibitors of HCV NS3 protease and polymerase telaprevir (VX950) and mericitabine (R7128) impaired HCV replication in 293T-4R/miR122 cells (Fig. 2F). These data demonstrate that HCVcc RNA replication in kidney-derived 293T-4R/miR122 cells is efficient and dependent on mechanisms similar to those in liver-derived Huh7.5.1 cells. Despite efficient entry and RNA replication of 293T-4R/miR122 cells infected with recombinant HCVcc, these cells did not release infectious virions, suggesting that kidney-derived cells lack factors required for viral assembly and release. Therefore, we aimed to reconstitute virus production by expression of HCV assembly factors. HCV production shares factors involved in very-low-density lipoprotein (VLDL) assembly, a process that occurs exclusively in hepatocytes (13, 14, 27). While the necessity of apolipoprotein B (ApoB) in HCV production is controversial (15), ApoE is known to be critical and is incorporated into the virion (26). We therefore expressed the most common isoform of ApoE (ApoE3) in 293T-4R/miR122 cells by using a lentiviral vector encoding human ApoE3 as described previously (23) and confirmed its expression by flow cytometry using an ApoE-specific antibody (Fig. 3A). We then infected 293T-4R/miR122/ApoE cells. Subsequently, the production and release of viral particles was assessed by incubating naive Huh7.5.1 cells with the supernatants from these cells. Indeed, 293T-4R/miR122/ApoE released infectious particles with similar electrophoretic mobility and replication kinetics as native HCVcc (Fig. 3B). We therefore concluded that expression of ApoE in kidney-derived 293T cells was sufficient to reconstitute virus assembly and release.

A.  

<table>
<thead>
<tr>
<th>Loci Tested</th>
<th>AMELO</th>
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<th>D13</th>
<th>D7</th>
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<tr>
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<td>8, 9, 12, 14</td>
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<td>293T cells</td>
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<td>8, 9, 12, 13, 14</td>
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B.  

<table>
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<th>OCLN</th>
<th>CLDN1</th>
<th>SR-BI</th>
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<td>SR-BI</td>
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</table>

C.  

**FIG 1** Expression of four HCV entry factors renders 293T cells highly permissive to HCVpp entry. (A) Short tandem repeat (STR) profile of the 293T cells used in this study (cell line authentication, LGC Standards) was performed as described previously (1). The names of tested loci are in bold, and peak positions from STR profile of 293T cells were compared to the LGC Standards database. (B) 293T cells (cultured in Dulbecco’s modified Eagle medium with high glucose; Life Technologies) were transduced with lentiviruses (as described in reference 3) to express given HCV entry factors. After transduction, cells were selected with 12 μg/ml of blasticidin for 2 weeks. Blasticidin-resistant cells were analyzed by flow cytometry using monoclonal antibodies (CLDN1 [11], OCLN [catalog no. 33–1500; Invitrogen], and SR-BI [Zahid et al., submitted for publication]) recognizing indicated entry factors. Entry factor-transduced cells (dark gray histograms) were compared to naive 293T cells (light gray histograms) and isotype control antibody (catalog no. 10400C; Life Technologies) (white histograms with dashed lines). The x axis shows fluorescence intensity; the y axis shows the number of events. (C) Transduced 293T cells were assessed for HCVpp entry by determining luciferase activity 72 h postinfection as previously described (35). Results were first normalized to vesicular stomatitis virus pseudoparticle entry (VSV-Gpp; produced as described in reference 29) and then compared to those obtained with Huh7.5.1 cells (cultured as described in reference 41). Results are means and standard deviations (SD) from three independent experiments performed in triplicate. Entry is relative to entry into Huh7.5.1 cells, and 100% relative infectivity is represented by a solid line. Statistical analysis for entry factor expressing cells relative to naive 293T cells was performed using the Student t test (*, P < 0.05).
FIG 2 293T-4R cells support robust HCV infection upon miR122 expression. (A) Stable 293T-4R cells described in the legend to Fig. 1 were challenged with HCVcc (JcR2a; produced as described in reference 38) or were mock infected, and luciferase activity was assessed 72 h postinfection as described previously (38). Results are means and SD, in relative light units (RLU), from three independent experiments performed in triplicate. (B) 293T-4R cells were stably transduced using miR122-encoding lentiviruses (catalog no. mh15049; ABM) and 2.5 μg/ml of puromycin-resistant cells were selected over 2 weeks. 293T-4R/miR122 cells and Huh7.5.1 cells were then infected with HCVcc or mock infected for 6 h. Infection was assayed by monitoring luciferase activity 72 h postinfection. Results are means and SD from three independent experiments performed in triplicate. Jc1, an HCVcc without a luciferase reporter (32), was likewise used to infect Huh7.5.1 and 293T-4R/miR122 cells, and its infectivity was assessed by limiting-dilution assay (TCID50) by detecting viral protein NS5A using immunohistochemistry, represented as gray bars (22). Results are expressed as means and SD from three independent experiments. (C) Northern blots of miR122 and miR-16, and U6 RNA as a loading control, extracted from 293T-4R cells, 293T-4R cells stably expressing miR122, and Huh7.5.1 cells as positive control. Northern blotting using a miR122-specific probe were performed as described previously (30). Oligonucleotide lengths (in nucleotides [nt]) are indicated on the left. (D) 293T-4R, 293T-4R/miR122, and Huh7.5.1 cells were incubated side by side with HCVcc (JcR2a), and luciferase activity was monitored every 24 h over a 72-h period. Results are means and SD from three independent experiments performed in triplicate. (E) 293T-4R/miR122 cells were preincubated for 1 h at 37°C with the indicated entry inhibitors, antivirals, or controls (20 μg/ml monoclonal antibodies [Mab], anti-CD81 [JS81; BD Biosciences], anti-CLDN1 [11], and anti-SR-BI [Zahid et al., submitted]; 1:200 dilution of polyclonal antibody [PAb] anti-ApoE [catalog no. 178479; Calbiochem]; 20 μM protease inhibitor telaprevir VX950; 1 μM polymerase inhibitor mericitabine R7128 [both synthesized by Acme Bioscience Inc.]; and 0.7% dimethyl sulfoxide [DMSO]) and then infected with HCVcc (JcR2a) in the presence of the given entry inhibitors or antivirals. Cell lysates were assessed for luciferase activity 72 h postinfection. Results are means and standard errors of the means from three independent experiments performed in triplicate. Values are relative to controls, and 100% relative infectivity is represented by a solid line. In panels A, B, and D, detection limits are represented by dashed lines. Statistical analysis relative to control was performed using the Student $t$ test (*, $P < 0.05$).
HCV particles as shown by a marked and highly significant increase in infectivity (as assessed by luciferase activity of JcR2a virus and TCID50 of Jc1 virus without a reporter gene) of the supernatant compared to the supernatant of 293T-4R/miR122 cells without ApoE expression (Fig. 3B). Although the production of infectious particles was lower than in Huh7.5.1 cells studied in side-by-side experiments, these data indicate that ApoE is a key factor for virus production in reconstituting the viral life cycle in nonhepatic cells. This diminished HCV production was not due to diminished replication levels, as ApoE-transduced cells had HCV replication levels similar to those of 293T-4R/miR122 cells prior to ApoE expression (data not shown). To test if HCV produced by these cells is reliant only on the human ApoE3 isoform or could use other forms of ApoE, we similarly transduced human ApoE2 and ApoE4 isoforms, as well as murine ApoE (Fig. 3C). Viruses produced from 293T cells expressing these ApoE isoforms and the mouse ortholog had similar infectivity compared to human ApoE3 isoform (Fig. 3D).

Focusing on the most common ApoE isoform (ApoE3), we further characterized the kinetics and attributes of these viruses. First, we confirmed that HCV particles from engineered 293T cells could establish infection by monitoring the increase in HCV genomes over time in Huh7.5.1 target cells after exposure to the supernatant of HCVcc-infected 293T-4R/miR122/ApoE cells (Fig. 4A). Next, we characterized the kinetics of HCV RNA production from infected 293T-4R/miR122/ApoE cells by infecting them with HCVcc (JcR2a), and 72 h postinfection, supernatants of infected 293T-derived cells were passaged onto naive Huh7.5.1 cells. At 72 h after infection was initiated, Huh7.5.1 cells were lysed and luciferase activity assessed. Results are means and SD from a representative experiment performed in triplicate. The dashed line represents the detection limit.
viruses (1/900 for Huh7.5.1-derived virus and 1/26,000 for 293T-4R/miR122/ApoE-derived virus). It should be noted that HCV particles produced from 293T-4R/miR122/ApoE cells proved to have a route of infection similar to that of liver-derived HCVcc, in that entry into Huh7.5.1 cells was neutralized by well-characterized HCV entry inhibitors, including CD81-, SR-BI-, CLDN1-, and ApoE-specific antibodies and erlotinib (Fig. 4C). Fractionating the virus by iodixanol density gradients revealed that the infectious virions produced from 293T-4R/miR122/ApoE cells have a buoyant density similar to those from Huh7.5.1 cells (Fig. 4D).

The data presented here demonstrate that trans-expression of OCLN, CD81, CLDN1, SR-BI, miR122, and ApoE endows 293T human kidney-derived cells with the capacity to support the complete HCV life cycle. Expression of four principal entry factors and miR122 generated cells with higher entry levels than and similar replication kinetics to those of extensively optimized Huh7.5.1 cells (4, 41). It should be noted in this context that the recently identified entry factor EGFR is also expressed in 293T cells (data not shown) (24, 40). We confirmed that expression of CLDN1 alone appears to be sufficient for infection of 293T cells (7) and expand these findings by showing that high-level expression of the four canonical HCV entry factors makes previously impenetrable cells fourfold more permissive than Huh7.5.1 cells. These observations were confirmed by HCVcc infection of 293T cells engineered to express miR122 in addition to variable sets of entry factors (data not shown). These results indicate that concomitant high-level expression of the four human entry factors is required for robust HCV entry into mouse hepatocytes in vivo (6). Since none of the identified entry factors are exclusively expressed in the liver, it is likely that the combined expression of these host factors at substantial levels allows the virus to productively infect the human liver, rather than a single liver-specific entry factor restricting HCV infection.

Investigators have shown that miR122 expression increases HCV replication in mouse embryonic fibroblasts and other hepatoma cell lines such as HepG2 cells (17, 21, 28). Furthermore, HEK-293 cells modified to express miR122 are capable of supporting HCV infection, but infection of mouse embryonic fibroblasts is partially dependent on the expression of miR122 (5). We demonstrate here de novo replication following an infection event of a nonhepatic cell line engineered to express HCV host factors. Our data also demonstrate that there is no restrictive factor of HCV entry and viral RNA replication that is present in 293T cells. HCV entry and replication in human blood brain barrier endothelial and neuronal cells have been studied extensively, but the exact mechanisms by which HCV enters these cells remain unclear. Understanding the molecular basis of HCV entry into nonhepatic cells is crucial for the development of novel strategies to combat HCV infection.

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FIG 4 Characterization of HCVcc derived from 293T-4R/miR122/ApoE cells. (A) Culture media from Jc1-infected 293T-4R/miR122, 293T-4R/miR122/ApoE, and Huh7.5.1 cells were passaged onto naive Huh7.5.1 target cells. Total RNA from these Huh7.5.1 target cells was extracted at the indicated time points, and HCV RNA was quantitated by reverse transcription-quantitative PCR (RT-qPCR) as described previously (11). Values were normalized to the value for the internal control gene (GAPDH gene). Results are means and SD from an experiment performed in quadruplicate. (B) HCV RNA production was measured by infecting 293T-4R/miR122, 293T-4R/miR122/ApoE, and Huh7.5.1 cells side by side with HCVcc (Jc1). RNA from supernatants of infected cells was extracted at the indicated time points, and HCV RNA was quantitated by RT-qPCR. Results are means and SD from an experiment performed in triplicate. (C) Culture media of infected 293T-4R/miR122/ApoE cells were harvested 72 h postinfection and passaged onto naive Huh7.5.1 cells that had been preincubated with either control IgG, DMSO, or the indicated entry inhibitors. Results are mean percentages of HCV infection (as assessed by luciferase activity) relative to the control and SD from a representative of two independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. The virus used was JcR2a with a TCID50 of 10^3 to 10^5/ml. (D) Density distributions of infectious 293T-4R/miR122/ApoE- and Huh7.5.1-derived HCVcc (Jc1) were determined by overlaying 0.5 ml of culture medium on a 5-ml, 4-to-40% iodixanol step gradient and ultracentrifuging samples for 16 h at 40,000 rpm on an SW-55 rotor (Beckman Coulter). Fractions were carefully harvested from the top of each tube, and density was determined by weighing 0.5 ml of each fraction. Each fraction was assayed for infectivity by TCID50 by detecting NS5a as described previously (22).
been described (9, 10). In contrast to the kidney-derived cells described here, HCV replication in blood-brain barrier endothelial cells occurred via a miR122-independent mechanism but at a diminished level (9). Thus, the cell lines developed in this study may be useful as a tool to further understand the molecular mechanisms of extrahepatic infection.

The production of HCV in 293T-4R/miR122/ApoE cells was diminished relative to that in HuH7.5.1 cells but markedly and significantly higher than that in cells without ApoE expression. This demonstrates that apart from ApoE, all the other factors necessary for the production of infectious particles are present in 293T cells, yet additional host factors may increase efficient production levels. The cell line generated in this study is likely to allow further discovery of the minimal set of host factors required for robust viral production. Additional relevant factors enhancing viral production may be ApoB (27), DGAT1 (13), or microsomal triglyceride transfer protein (MTP) (12, 14). Notably, ApoE has recently been demonstrated to be essential for virus production; ApoE-deficient mouse hepatocytes with trans expression of HCV RNA and proteins along with ApoE are able to produce high levels of infectious virions (23).

In summary, this study demonstrates that a small set of defined host factors is sufficient to reconstitute the complete viral life cycle in nonhepatic cells. These results advance our knowledge of tissue-specific factors for HCV infection and provide novel tools to elucidate host and restriction factors for the HCV life cycle.

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REFERENCES